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Long-Term Effect of Mutagenic DNA Repair on Accumulation of Mutations in *Pseudomonas syringae* B86-17

Shouan Zhang[1] and George W. Sundin[2,3,4]*

National Center for Agricultural Utilization Research, USDA Agricultural Research Service, Peoria, Illinois,[1] and Program in Genetics,[2] Center for Microbial Ecology,[3] and Department of Plant Pathology,[4] Michigan State University, East Lansing, Michigan

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Forty replicate lineages of *Pseudomonas syringae* B86-17 cells expressing the *rulAB* mutagenic DNA repair (MDR) determinant or the *rulB*::Km MDR-deficient mutant GWS242 were passaged through single-cell bottlenecks (60 cycles), with a UV radiation (UVR) exposure given to half of the lineages at the beginning of each cycle. After every 10th bottleneck cycle, single-colony isolates from all 80 lineages were subjected to 39 phenotypic screens, with newly arising mutations detected in 60 and 0% of UVR-exposed or non-UVR-exposed B86-17 lineages, respectively, by the 60th cycle. Cellular fitness, measured as growth rate in a minimal medium, of UVR-exposed lineages of both B86-17 and GWS242 after 60 cycles was not significantly different from that of the ancestral strains. Although UVR exposure and MDR activity increased the occurrence of mutations in cells, a significant reduction in overall fitness was not observed.

Mutators are cells with increased genomic mutation rates, typically due to inactivation of DNA mismatch repair systems. Mutators occur at unexpectedly high percentages in natural populations of bacterial pathogens, including *Escherichia coli*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, and *Salmonella* (16, 19, 23). Indeed, mutators are favored by changing environmental conditions (31), as might be experienced by pathogenic organisms during colonization and subsequent initiation of disease in a susceptible host. Mutator strains have arisen in experimental evolving populations (18, 21, 26) and are competitively favored in mixed cultures when initially present at frequencies above 5×10^{-5} (1). Although the majority of new mutations generated within mutator cells are thought to be deleterious, these strains can be retained in populations due to an increased likelihood of generating and thus becoming physically linked with any rare beneficial mutations arising within the mutator cell (2).

Since all natural organisms are not mutators, it is clear that this lifestyle is not favored in all environments. For example, the mutation rate of organisms inhabiting constant environments is typically low (3). Additionally, in the absence of recombination, smaller populations are subject to eventual extinction due to the accumulation of deleterious mutations by genetic drift (9, 20). In experimental systems, passage of mutator *E. coli* and yeast populations through repeated genetic bottlenecks resulted in significant deleterious mutation accumulation and the extinction of some lineages through a process termed mutational meltdown (7, 33). However, if a mutator can revert to a nonmutator form once an adapted fitness state is reached, theoretical studies suggest that this fitness state can be maintained (30); reversion is ecologically relevant during pathogenesis, for example, a situation in which mutator strains may be favored only in certain environments and disfavored in others (8).

Transient mutator status has been argued to represent a better cellular strategy in terms of adaptation to environmental stress (24). One such transient mutator strategy is mutagenic DNA repair (MDR) or translesion synthesis, which is encoded by determinants, such as *E. coli umuDC*, that are regulated by the cellular SOS DNA damage response (29). *umuDC* and its bacterial homologs encode specialized DNA polymerases (Pol V) that can replicate past lesions that are normally not repairable by other cellular DNA repair mechanisms (6). These inducible systems therefore only transiently increase the cellular mutation rate; this function is accompanied by a second, selectable function in that most MDR determinants confer tolerance to UV radiation (UVR). However, the effect of MDR on the accumulation of deleterious mutations has not been established. A known ecological role for MDR-mediated UVR tolerance has only been demonstrated for *rulAB*, an MDR determinant in *P. syringae* pv. syringae, a plant pathogen that inhabits sunlight-exposed plant leaf surfaces (12, 27). Our long-term goal is to understand the impact of MDR-mediated genetic alterations on cellular evolution in *P. syringae* and other bacterial populations. Here, we used single-cell bottleneck experiments to examine the accumulation of gene-inactivating mutations in a *P. syringae* pv. syringae strain, B86-17, encoding *rulAB*, and the near-isogenic *P. syringae* pv. syringae *rulB*::Km mutant GWS242 in the presence or absence of regular UVR exposure.

Long-term bottleneck experiments incorporating UVR exposure. The experiments were initiated with *P. syringae* pv. syringae B86-17, a wild-type strain that encodes the *rulAB* MDR determinant on an indigenous plasmid (28), or the insertional mutant *P. syringae* subsp. *syringae* GWS242 (B86-17 *rulB*::Km) (12). The UVR survival of GWS242 is reduced approximately three- to fivefold from that of B86-17, and UVR mutability is also reduced, although not completely eliminated (12). Single colonies of B86-17 and GWS242 were inoculated

* Corresponding author. Mailing address: Michigan State University, 103 Center for Integrated Plant Systems, East Lansing, MI 48824-1311. Phone: (517) 355-4573. Fax: (517) 353-5598. E-mail: sundin@msu.edu.

TABLE 1. Phenotypes (loss of) examined to assess the accumulation of mutations in replicate lineages of *P. syringae* pv. syringae B86-17 and GWS242

Phenotype(s) examined (n^a)	Substances or responses assayed
Auxotrophic requirements (19)	Alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine
Carbon source utilization (12)	Betaine, choline, citrate, erythritol, glycerol, β-hydroxybutyrate, inositol, lactate, quinic acid, sorbitol, sucrose, trigonelline
Loss of function (2)	Hypersensitive response on tobacco, motility
Physiological responses (6)	Aesculin hydrolysis, arbutin hydrolysis, alteration of colony morphology, fluorescence on King's B medium, gelatin liquefaction, levan production from sucrose

[a] *n*, no. of tests.

into mannitol glutamate broth (10) containing 0.25 g of yeast extract per liter (MGY) and grown overnight. Appropriate dilutions of these cultures were plated onto MGY medium, and 40 single colonies of B86-17 and GWS242 were used to initiate independent lineages that were propagated by streaking onto MGY medium. Twenty lineages each of B86-17 and GWS242 were subjected to UVR irradiation (254-nm UVC wavelength, 30 J m^{-2}) immediately after streaking for each cycle. The UVC dose resulted in cell survival levels of approximately 40 to 50% for B86-17 and 10 to 20% for GWS242, and is sufficient to activate expression of the *rulAB* determinant (12). An additional 20 lineages each of B86-17 and GWS242 were propagated identically, except these lineages were not subjected to the UVC radiation dose. Each experimental cycle consisted of propagation by streaking onto fresh MGY medium, UVC irradiation (for half of the lineages), and incubation in darkness at 25°C for 72 h. We selected the furthest single colony on the plates for the next cycle, and the experiment continued in this manner for a total of 60 cycles. A portion of each of the 40 initial colonies from each strain (B86-17 and GWS242) was amplified on MGY and stored in 15% glycerol at −70°C for use in later phenotypic analyses; single-colony isolates from each of the 80 total lineages (B86-17 and GWS242; UVC irradiated and nonirradiated) were likewise stored after 10, 20, 30, 40, 50, and 60 cycles.

Phenotypic analysis of mutation accumulation. We used 39 phenotypic tests to assay for mutations arising during the experiment in each of the 80 bacterial lineages propagated in this study (Table 1). We examined isolates from each lineage recovered after every 10th cycle. Auxotrophs were initially identified by an inability to grow on unsupplemented mannitol glutamate minimal medium; auxotrophic requirements were identified by first adding amino acid supplements in groups of three and then adding individual amino acids. All amino acids were examined except glutamic acid, which is a component of mannitol glutamate medium. Carbon sources were filter sterilized and added at 0.1% (wt/vol) to Ayers et al. minimal salt medium (25). Bacterial strains were streaked onto plates and incubated at 28°C for 7 days; growth was compared to that on plates without added carbon sources. Fluorescence on King's B medium (14), gelatin liquefaction, hydrolysis of esculin and arbutin, levan production (25), and motility on "swim agar" plates (7) were analyzed as previously described. The hypersensitive reaction (HR) tests the ability of *P. syringae* pv. syringae B86-17 and GWS242 (both plant pathogens on bean) to elicit a defense response in the non-host plant tobacco and is dependent upon a functional type III secretion system and functional effector proteins (15). Briefly, cell suspensions (~10^8 CFU ml^{-1}) were inoculated into intraveinal sections of *Nicotiana tabacum* leaves with a blunt syringe; the presence of localized cell death at the inoculation site 24 h after inoculation was scored as a positive reaction. Colony morphology of derived strains was assessed visually by comparison with ancestral strains on MGY plates.

During each of 60 cycles, colonies chosen had grown from 1 cell to approximately 1.7×10^8 cells, or ca. 27 generations per cycle (1,620 generations total for the experiment). If UVR-exposed *P. syringae* pv. syringae B86-17 cells behaved like mutators, then we would expect to observe an increase in mutations in UVR-exposed cells relative to nonexposed cells over the course of the experiment. For example, Funchain et al. (7) tracked 100 mutator (*mutS*) lineages of *E. coli* J93 and detected at least one mutation in 38 and 67% of the lineages after 10 and 20 cycles, respectively, while mutations were not observed in wild-type lineages. Following 60 cycles, 99% of the J93 lineages had at least 1 detectable mutation with a mean of 4 to 5 mutations per lineage (7).

In our experiment, mutations in the UVR-exposed B86-17 lineages were not observed until after cycle 20 (Table 2). The increase in strains with mutations and in the total number of mutations occurred more sharply between cycles 20 and 60; however, 8 lineages (40%) with no detectable mutations remained after cycle 60 (Table 2). No mutations were observed in any lineage (B86-17 or GWS242) without UVR exposure after 60 cycles (Table 2). Strain GWS242, which has an insertional mutation within the plasmid-encoded *rulB* gene, was previously reported to possess a low but consistently detectable UVR mutability frequency, which was surmised to be associated with a chromosomal MDR locus (12). Six of twenty lineages of UVR-exposed GWS242 had detectable mutations after cycle 60, with a lower number of overall mutations than B86-17 (Table 2).

Among UVR-exposed experimental lineages, we detected a total of 30 mutations among 12 lineages of B86-17 and 15 mutations among 6 lineages of GWS242 in this experiment. The overall pattern of phenotypes affected was not distinctly different for both B86-17 and GWS242. The most common phenotypic differences identified were alterations in colony morphology (eight B86-17 lineages and three GWS242 lineages), defects in levansucrase activity (five B86-17 lineages and four GWS242 lineages), and loss of motility (four B86-17 lineages and one GWS242 lineage). Defects in the utilization of various carbon sources constituted 11 mutations distributed

TABLE 2. Accumulation of mutations in *P. syringae* pv. syringae B86-17 and GWS242 lineages with or without periodic UVC exposure

Strain	UVC trt.[a]	Cycle	No. of lineages with the following number of mutations[b]:									
			0	1	2	3	4	5	6	7	8	9
B86-17	None	0	20	0	0	0	0	0	0	0	0	0
		10	20	0	0	0	0	0	0	0	0	0
		20	20	0	0	0	0	0	0	0	0	0
		30	20	0	0	0	0	0	0	0	0	0
		40	20	0	0	0	0	0	0	0	0	0
		50	20	0	0	0	0	0	0	0	0	0
		60	20	0	0	0	0	0	0	0	0	0
B86-17	30 J m^{-2}	0	20	0	0	0	0	0	0	0	0	0
		10	20	0	0	0	0	0	0	0	0	0
		20	17	1	1	0	0	0	1	0	0	0
		30	14	3	2	0	0	0	0	1	0	0
		40	10	6	2	1	0	0	0	0	0	1
		50	10	5	2	1	0	1	0	0	0	1
		60	8	6	2	2	0	1	0	0	0	1
GWS242	None	0	20	0	0	0	0	0	0	0	0	0
		10	20	0	0	0	0	0	0	0	0	0
		20	20	0	0	0	0	0	0	0	0	0
		30	20	0	0	0	0	0	0	0	0	0
		40	20	0	0	0	0	0	0	0	0	0
		50	20	0	0	0	0	0	0	0	0	0
		60	20	0	0	0	0	0	0	0	0	0
GWS242	30 J m^{-2}	0	20	0	0	0	0	0	0	0	0	0
		10	20	0	0	0	0	0	0	0	0	0
		20	17	2	1	0	0	0	0	0	0	0
		30	16	0	4	0	0	0	0	0	0	0
		40	16	0	3	1	0	0	0	0	0	0
		50	15	0	3	0	2	0	0	0	0	0
		60	14	1	3	0	2	0	0	0	0	0

[a] Cultures listed as 30 J m^{-2} were irradiated with this dose of UVC following transfers initiating the next cycle. trt., treatment.

[b] Twenty lineages of each strain/UVC treatment were examined. A total of 45 mutations were identified by using the phenotypic screens described in Table 1.

among 8 B86-17 lineages and 7 mutations distributed among 4 GWS242 lineages.

Growth rate comparisons. Prolonged passage through bottlenecks is associated with loss of fitness in mutator and nonmutator lineages (11). We assessed fitness in our experiments by comparing the growth rate (measured as cell doubling time) of an isolate from each of the 20 derived lineages per treatment recovered at cycle 60 with the appropriate ancestral strains stored at the initiation of the experiment. All strains were initially removed from storage at −70°C and grown overnight in Luria-Bertani (Difco) broth; cells were then diluted 1:50 into 25 ml of MGY broth contained in a 50-ml flask and incubated at 28°C with shaking at 250 rpm. After 160 min, samples were taken every 15 min for 2.5 h; cell numbers were estimated by assessing the turbidity at 600 nm. Standard curves relating turbidity values to cell numbers were generated, and regressions were made based on growth curve data for each experimental strain. Doubling time was determined for each strain from the line drawn through the points during exponential growth. The mean doubling time of cycle 60 strains was slightly increased in all experimental groups but not significantly different from that of ancestral (cycle 0) strains (Table 3), indicating that the prolonged bottlenecks and in some cases regular UVR exposure did not exert a negative effect on the growth rate of cultures.

MDR determinants enhance UVR survival on host cells via translesion synthesis, an enzymatic mechanism that replicates past lesions that are otherwise not repairable by the available repertoire of DNA repair systems (32). A by-product of translesion synthesis is an increase in the cellular mutation rate, since this is a low-fidelity replication mechanism. Unlike mutators that affect DNA mismatch repair, MDR systems are inducible and may not mutate the genome randomly. Biochemical studies have shown that mutational hot spots exist and that local DNA sequence context plays an important role in the type and frequency of mutational events (summarized in reference 5). A critical factor is that the location of UVR-induced mutational hotspots is not predictable based on knowledge of the DNA sequence (5, 17). Echols (4) suggested that the role of MDR in bacteria could be that of UVR protection, a potential role in adaptive evolution, or both. Our previous results demonstrated the importance of MDR-mediated UVR protection for *P. syringae* and other *Pseudomonas* spp. (12, 13, 28, 34), and this study revealed the occurrence of mutations in UVR-exposed lineages, without apparent effect on overall fitness (measured as growth rate). In contrast to results in a previous study utilizing an *E. coli* mutator cell line (7), none of our lineages became extinct, and the growth rate of lineages after cycle 60 did not differ from the growth rate at cycle 0. Thus, the transient mutator strategy of UVR-induced MDR, although resulting in the generation of deleterious mutations in some lineages, may represent an effective ecological strategy in adaptive evolution in response to environmental stress. It was recently discovered that RulB, UmuC, and other bacterial MDR proteins belong to the Y family of DNA polymerases that occur from bacteria to humans (22). This knowledge indicates that an increased ecological understanding of these polymerases could have far-reaching biological implications.

TABLE 3. Mean doubling time (min) of *P. syringae* pv. syringae B86-17 and GWS242 lineages after propagation for 60 cycles of single colony streaking

Strain	UVC trt.[b]	Mean doubling time[a]	
		Cycle 0	Cycle 60
B86-17	None	37.0	37.5_{NS}[c]
B86-17	30 J m^{-2}	36.1	39.4_{NS}
GWS242	None	42.8	44.2_{NS}
GWS242	30 J m^{-2}	43.7	45.0_{NS}

[a] Data from each of the 20 lineages (cycle 0 and cycle 60) per treatment are included in each mean.

[b] Cultures listed as 30 J m^{-2} were irradiated with this dose of UVC following transfers initiating each cycle. trt., treatment.

[c] NS, not significant ($P > 0.05$), following an analysis of variance and data mean comparison using the Student *t* test.

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